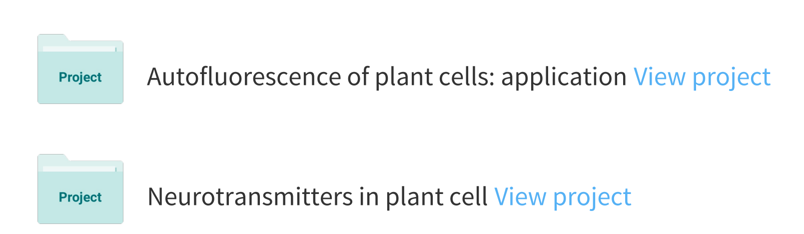
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Study of the mechanism of permeabilization of lecithin liposomes and rat liver mitochondria by the antimicrobial drug triclosan

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Abbreviations: TCS – triclosan; CsA - cyclosporin A; LUV - large unilamellar vesicles; MPT – mitochondrial permeability transition; SRB - sulforhodamine B

ABSTRACT

The effect of the antimicrobial compound triclosan (5-chloro-2'-(2,4-dichlorophenoxy)phenol;) on the permeability of lecithin liposomes and rat liver mitochondria was studied. It was found that triclosan was able to increase nonspecific permeability of liposomes in a dose-dependent manner, which was detected by the release of the fluorescent probe sulforhodamine B (SRB) from vesicles. A partial release of SRB occurs instantly at the moment of triclosan addition, which is followed by a slow leakage of the dye. The triclosan-induced release of SRB from liposomes grew as pH of the medium was decreased from 9.5 to 7.5. As revealed by the laurdan generalized polarization (GP) technique, triclosan increased laurdan GP in lecithin liposomes, indicating a decrease in membrane fluidity. Measurements of GP as a function of fluorescence excitation wavelength gave an ascending line for triclosan-containing liposomes, which can be interpreted as phase heterogeneity of the lipid/triclosan system. Dynamic light scattering experiments also showed that at a high triclosan-to-lipid molar ratio (~0.5), a population of smaller light-scattering particles (~0.4 of the size of liposomes) appear in the system. Experiments with rat liver mitochondria demonstrated that triclosan (10–70 μM) induced a high-amplitude cyclosporin A-insensitive swelling of the organelles accompanied the release of cytochrome *c*. On the basis of the results obtained, possible mechanisms of the toxic effect of triclosan in eukaryotic cells are discussed.

Keywords: triclosan, membrane permeabilization, liposomes, mitochondria

1. Introduction

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) (TCS) is a synthetic drug with a broad spectrum of antimicrobial activity [1]. The antibacterial effect of TCS has been well studied and is generally considered to be associated with the inhibition of the enoyl-acyl carrier protein reductase (ENR), a key enzyme in the biosynthesis of fatty acids. As a result of ENR suppression, the synthesis of membrane phospholipids and proliferation of microbial cells are inhibited [2,3].

It has long been believed that TCS is harmless to mammals, since they do not have the ENR enzyme. However, evidence has been recently accumulated that toxic effects of TCS are also manifested in eukaryotes [1,4-7]. It is assumed that in the eukaryotic cells, TCS action is primarily directed at the membrane and membrane-associated proteins. It has been found that TCS affects the functioning of a number of receptors, membrane enzymes (NADPH oxidase), and intracellular Ca^{2+} channels (ryanodine receptors) [11-13]. The studies on mitochondria have shown that TCS uncouples oxidative phosphorylation and inhibits activity of the mitochondrial respiratory chain complexes [14-16]. All these effects can lead to a disturbance of intracellular ion homeostasis, enhanced generation of reactive oxygen species (ROS), oxidative stress, and apoptotic cell death [8,17,18].

In addition to its effects on the protein component of membranes, TCS, as a hydrophobic molecule, can also directly affect properties of the phospholipid bilayer. Experiments with artificial membranes formed from synthetic phospholipids have shown that TCS can decrease temperature of the main phase transition and induce formation of a hexagonal-H_{II} phase [19,20]. This is probably due to the fact that TCS is incorporated in the upper region of the phospholipid membrane, with its hydroxyl group residing in the vicinity of the C=O/C2 carbon atoms of the phospholipid acyl chain, with the rest of the TCS molecule being oriented nearly perpendicular to phospholipid molecules [21]. It can be assumed that such an orientation of the TCS molecule in the bilayer will lead to a disturbance of phospholipid packing, which may be accompanied by the appearance of lipid pores and the corresponding changes in the permeability of the membrane to ions and larger molecules. These changes can, in their turn, be the cause of cell death. At concentrations higher than 10^{-4} M, for example, TCS was found to induce lysis of some microorganisms, which was attributed to the disorder of their membrane structure [20,22]. At the same time, it is important to note that mammalian cells can be even more sensitive to TCS than cells of some microorganisms [14,20,23].

In mitochondria, opening of lipid pores and nonspecific transitions in membrane permeability may be involved in the mechanisms of the apoptotic cell death. Earlier we showed that the formation of the mitochondrial palmitate/Ca^{2+}-induced pore, which can be considered as

a fast-tightening lipid pore, could induce a high-amplitude swelling of mitochondria and the release of proapoptotic proteins from the organelles [24-26]. On the basis of the known effects of TCS on membranes formed from synthetic phospholipids [19,20], one can assume that at moderate concentrations, TCS will also influence the permeability of both the artificial membranes formed from natural phospholipids (lecithin) and the biological (mitochondrial) membranes. Thus, the objectives of the present study were (1) to examine the ability of TCS to cause permeabilization of liposomal membranes; (2) to study the mechanism by which TCS permeabilizes membranes of lecithin liposomes; and (3) to evaluate the possibility of this mechanism to work in the inner membrane of rat liver mitochondria.

2. Materials and Methods

2.1. Materials

Medium components, inorganic chemicals, triclosan, sulforhodamine B (SRB), CsA, phosphatidylcholine (PC) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Laurdan was purchased from Fisher Scientific (Waltham, Massachusetts, USA; Invitrogen™).

2.2. Isolation of rat mitochondria

Mitochondria were isolated from the liver of Wistar rats (220-250 g) by differential centrifugation as described [27]. The homogenization buffer contained 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM Hepes/KOH buffer, pH 7.4. Subsequent centrifugations were performed in the same buffer, except that, instead of EDTA, 100 μM EGTA was used. Final suspensions contained 70–80 mg of mitochondrial protein/mL, as determined by the Lowry method [28].

2.3. Mitochondrial swelling

The swelling of mitochondria (0.4 mg/mL) was measured as a decrease in A_{540} in a stirred cuvette at room temperature (~22°C) using a USB-2000 spectroscopy fiber-optic system (Ocean Optics, USA). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 10 μM EGTA, 1 μM rotenone, and 10 mM Hepes/KOH buffer, pH 7.4. The rate of swelling ($V_{max} = \Delta A_{540}$/min per mg protein) was calculated as a change in absorbance within the first 30 sec from the beginning of the high-amplitude swelling.

2.4. Determination of cytochrome c release

The amount of cytochrome *c* released from mitochondria was determined spectrophotometrically according to the method described in [29]. Isolated liver mitochondria

(~1.0 mg/mL) were incubated in the medium containing 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 10 μM EGTA, 1 μM rotenone, and 10 mM Hepes/KOH buffer, pH 7.4 for 30 min at 25°C in the absence or presence of triclosan. After incubation, mitochondria were centrifugated (10000 g; 30 min), and the resulting supernatant was filtered through a 0.2 μm Millipore membrane. The optical density of the cleared supernatant was measured at 414 nm using a Multiskan GO Plate reader (Thermo, Finland). The concentration of cytochrome *c* was calculated using a calibration curve, with the cytochrome c dissolved in the incubation medium as a standard.

2.5. Confocal microscopy imaging of mitochondria

Mitochondria (0.4 mg protein/mL) were incubated for 10 min in the medium containing 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 10 μM EGTA, 1 μM rotenone, and 10 mM Hepes/KOH buffer, pH 7.4 and 0.5 μM Mitotracker red. The samples (10 μL of mitochondrial suspension) were placed on glass slides and inspected under a scanning confocal microscope Leica TSC SP5 (Germany) with a PL APO 63x/1.40 Oil *λ*bl (lambda blue) objective. Fluorescence was excited by a laser with λ = 543 nm and registered in the spectral range channel of 591 - 716 nm.

2.6. Electron microscopy of rat liver mitochondria

For electron microscopy examination, samples of mitochondria were fixed for 2 h in 2.5% glutaraldehyde dissolved in the medium containing 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 10 μM EGTA, 1 μM rotenone, and 10 mM Hepes/KOH buffer, pH 7.4, and post-fixed in 1% osmium tetroxide. Samples were embedded in Epon 812. Microscopic sections were sliced on an LKB-III microtome and stained with lead and uranylacetate. Electron microscopy was performed with a Tesla BS-500 microscope.

2.7. Preparation of liposomes

Liposomes (large unilamellar vesicles, LUV) were obtained by a conventional extrusion technique [30]. Dry egg phosphatidylcholine (PC) (lecithin 7.5 mg) was hydrated for several hours with periodical stirring in 0.75 mL of a buffer containing 10 mM Tris/HCl (pH 7.5), 40 mM KCl, and 50 μM EGTA. After five cycles of freezing/thawing at -20/+30°C, the suspension of multilamellar liposomes was pressed 11 times through a 0.1 μm polycarbonate membrane using an Avanti microextruder (Avanti Polar Lipids, Birmingham, AL). All operations (excluding the freezing/thawing procedure) were carried out at the room temperature.

2.8. Preparation of liposomes loaded with sulforhodamine B

Liposomes loaded with sulforhodamine B (SRB) were prepared from egg PC by a procedure similar to that described above except that (1) the buffer for lipid hydration contained 50 mM SRB instead of 40 mM KCl; and (2) after extrusion, liposomes were applied onto a Sephadex G-50 column to remove the external SRB. The buffer for gel filtration contained 10 mM Tris/HCl (pH 7.5), 50 μM EGTA and 40 mM KCl.

The release of SRB was evaluated by the increase in its fluorescence as described earlier [30]. The medium contained 10 mM Tris/HCl (pH 7.5), 50 μM EGTA, and 40 mM KCl. Fluorescence was measured using a USB-2000 spectroscopy fiber-optic system (Ocean Optics Inc., USA) (excitation at 565 nm, emission at 586 nm). The amount of SRB released was determined 5 min after the addition of TCS (no SRB release was observed prior to the addition of TCS). The total release of the dye was evaluated by the addition of 0.1% Triton X-100. The concentration of SRB in the external buffer was calculated using a calibration curve.

2.9. Measurements of laurdan generalized polarization (GP)

Laurdan is an environment-sensitive fluorescent probe, which is conventionally used for monitoring phase state of lipid membranes. We measured laurdan fluorescence using a Cary Eclipse spectrofluorimeter. The excitation wavelength for laurdan ranged from 320 to 400 nm. Emission wavelengths, corresponding to the blue and red peaks of laurdan in lecithin liposomes, were 430 and 490 nm. The generalized polarization (GP) was defined as GP = $(I_{430}-I_{490})/(I_{430}+I_{490})$ where I_{420} and I_{490} are the emission intensities at 430 and 490 nm, respectively [31,32]. GP can theoretically assume values from +1 (being most ordered) and −1 (being least ordered). In the experiments with laurdan-containing lecithin liposomes (laurdan/lipid molar ratio 1:200), the suspension of vesicles was added to a buffer containing 40 mM KCl, 50 μM EGTA and 10 mM Tris/HCl (pH 7.5) (the final concentration of lipid 50 μM) [33], and laurdan fluorescence was measured at 37°C before and after various experimental additions.

2.10. Dynamic light scattering

The size of particles in a LUV suspension was measured by dynamic light scattering (DLS) at 25°C using a Zetasizer Nano ZS device (Malvern Instruments Ltd.) [24]. LUV were prepared as described above. The concentration of phospholipid in samples was 50 μM. The medium contained 10 mM Tris/HCl buffer (pH 7.5), 50 μM EGTA, and 40 mM KCl.

2.11. Statistical analysis

The data were analyzed using the GraphPad Prism 5 and Excel softwares and were presented as means ± SEM of three to seven experiments. Statistical differences between the means were determined by a two-tailed *t*-test; $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. TCS induces release of SRB from lecithin unilamellar liposomes

Figure 1 shows the effect of TCS on the membrane permeability of lecithin unilamellar liposomes loaded with the fluorescent probe SRB. As can be seen in the figure, the addition of 26 μM TCS (7.5 μg/mL) resulted in an immediate partial release of SRB from liposomes. Following the initial release, the concentration of the dye in the buffer gradually increased.

The effect of TCS on the permeability of liposomes to SRB was investigated in more detail. Figure 1B shows dependence of SRB release on the concentration of TCS. As seen in the figure, the dye began to leak from liposomes at a TCS concentration of 8.5 μM (2.5 μg/mL). When the molar ratio of TCS to lipid in the system reached ~1.7 (34 μM or 10 μg/mL TCS), an almost complete release of SRB from liposomes was observed. The TCS-induced liposome permeabilization depended on pH of the incubation buffer (Fig. 2). One can see that lowering pH from 9.5 to 7.5 resulted in an increased TCS-induced release of SRB from vesicles.

3.2. TCS-induced changes in liposomal membranes revealed by laurdan fluorescence

It can be assumed that the mechanism of liposome permeabilization by TCS is related to changes caused by the anti-bacterial agent in the lipid bilayer of liposomal membranes. As revealed earlier by differential scanning calorimetry, TCS shifted the temperature of the main phase transition and gave rise to polymorphic phase transitions in the synthetic phospholipid systems [19,20]. In the present work, we examined effects of TCS on the state of lecithin membranes using the fluorescent probe laurdan.

Laurdan is an environment-sensitive fluorescent probe, which is conventionally used for monitoring phase state of lipid membranes. Generalized polarization (GP) of laurdan, an indicator defined as the relative difference between fluorescence intensities at two wavelengths (red and blue peaks), reflects hydration of membranes and mobility of water molecules in the region of lipid heads – parameters closely related to the phase state of lipid bilayer. Measured as a function of excitation wavelength (λ_{ex}), GP also provides information on heterogeneity of lipid environment of laurdan and can indicate coexistence of different phases [34,35]. The GP-vs-λ_{ex} line is horizontal in the gel phase, descending in the liquid-crystalline and liquid-ordered phases, and ascending when separate domains of different phases coexist [36-38].

The emission spectra of laurdan fluorescence in lecithin liposomes in the absence and presence of TCS are shown in Fig. 3A. It can be seen that TCS reduced the intensity of the red peak of the laurdan spectrum, thus, increasing laurdan GP (Fig. 3A, insert). This indicates that the membrane becomes less fluid. Measuring GP as a function of λ_{ex} showed that in the absence of TCS, GP values were negative and declined as λ_{ex} was increased (Fig. 3B) – reflecting the fact that the membrane of lecithin liposomes was in a homogeneous liquid-crystalline phase. After the addition of 34 μM TCS, the GP-vs-λ_{ex} line became an ascending function of λ_{ex}, indicating a phase or domain heterogeneity in the system.

3.3. Effect of TCS on the size of lecithin liposomes

Permeabilization of liposomes that we observed in the experiments with SRB and changes in the state of liposomal membranes revealed with laurdan suggested that liposomes might undergo structural rearrangements accompanied by a loss of membrane integrity. Such rearrangements could affect the size of liposomes or result in the appearance of other lipid and/or lipid/TCS structures – for example, if TCS would induce non-bilayer phase transitions in lecithin. To check this supposition, we assessed liposome size using the DLS technique.

The results of DLS measurements of lecithin liposomes in the absence and presence of TCS are given in Table 1. One can see that 17 μM TCS did not affect average hydrodynamic diameter of particles in the suspension. A higher concentration of TCS (34 μM) caused a slight increase in the diameter. At the same time, the addition of 51 μM TCS (to the TCS-to-lipid molar ratio > 1) resulted in the appearance of a second population of light-scattering particles, whose average hydrodynamic diameter was substantially smaller than the initial diameter of liposomes.

3.4. TCS induces cyclosporin A-insensitive swelling of rat liver mitochondria

As mentioned above, the membranotropic effects of TCS leading to destabilization of bacterial cell membranes occur at concentrations much higher than those used in the present study. The question arises whether low concentrations of TCS used in our experiments can permeabilize natural, e.g., mitochondrial membranes.

As shown in Fig. 4A, 34 μM TCS induced a high-amplitude swelling of rat liver mitochondria. The mitochondrial swelling occurred immediately after the addition of TCS, without a lag-period. The maximal swelling rate was observed at TCS concentrations above 50 μM (Fig. 4B). Fig. 4C shows typical electron micrographs of normal mitochondria with a dense matrix and visible cristae in comparison with organelles treated with TCS (51 μM). In the latter case, one can see a typical picture of mitochondrial swelling: (1) the size of mitochondria is much larger; (2) the organelles lose their outer membrane; (3) the electron density of the

mitochondrial matrix is lowered and (4) cristae disappear. The assessment of average hydrodynamic diameter of mitochondria in the absence and presence of 51 µM TCS gave values of 0.54±0.09 and 0.94±0.09 µm respectively.

As seen in Fig. 5, the addition of TCS to the suspension of mitochondria resulted in the release of cytochrome *c*, a proapoptotic protein.

It is known that some agents altering the membrane surface properties of mitochondria (e.g., otobaphenol, magnesium ions and/or oxidized cytochrome c or dextran [39-41] can cause their aggregation, which would complicate monitoring of mitochondrial swelling by the changes in their absorbance. Fig. 6 demonstrates that under our conditions, mitochondria did not form aggregates – both in the absence and presence of TCS.

The TCS-induced swelling of mitochondria was insensitive to the specific inhibitor of mitochondrial permeability transition (MPT) pore cyclosporin A (CsA) (Fig. 7A). This suggests that the TCS-induced mitochondrial permeabilization is not due to the opening of an MPT pore and occurs by another mechanism.

As shown earlier, TCS caused uncoupling of mitochondrial respiration and oxidative phosphorylation, as well as inhibition of the respiratory chain complex II activity. Figure 7B demonstrates that mitochondrial swelling induced by TCS occurred both in the absence and presence of respiratory substrates. However, it should be noted that in the presence of succinate as a respiration substrate, the rate of swelling was significantly higher.

4. Discussion

By now, a lot of data on the pharmacology and toxicology of TCS *in vivo* have been accumulated (see review [1]). Until recently, however, the data on the effect of this drug upon eukaryotic cells and organelles have been scarce and fragmentary. In general, it has been believed that the negative effects of TCS on mammalian cells are associated with its membranotropic action [20]. TCS is a hydrophobic compound, which means it will be incorporated into the lipid bilayer of biological membranes and affect their properties. Indeed, TCS was found to modulate the activity of membrane receptors and channels and influence the lipid component of membranes [6-10,17-21,42]. At concentrations above 10^{-4} M, TCS was shown to induce the release of intracellular enzymes from the cytoplasm, as well as lysis of cells [14,20,22]. At concentrations of ~10^{-6} M, TCS was also shown to act as a protonophore, inducing uncoupling of oxidative phosphorylation in mitochondria [14-16]. In the present work, we have established that, in addition to these effects, TCS is able to permeabilize both artificial (lecithin) and natural (mitochondrial) membranes. The permeabilization occurs at TCS concentrations comparable to those used for the inhibition of the bacterial ENR activity (the

minimal inhibitory concentrations of TCS are in the range of 10^{-6}-10^{-5} M [20]). At these concentrations, TCS seems to cause changes in the lipid bilayer, which are accompanied by a transient permeabilization of the membrane.

Study of the dynamics of TCS-induced permeabilization of liposomes have shown an instant partial release of SRB right after the addition of TCS followed by a gradual slow leakage of the dye (Fig. 1A). Obviously, the initial SRB release coincides with massive incorporation of TCS into liposomal membranes. The logarithm of TCS octanol/water partition coefficient is about 5 [43,44], so the content of TCS in the lipid phase should be relatively high under these conditions. With the content of TCS in the membrane high enough, one could expect changes in the basic properties of lipid bilayer or even formation of non-bilayer phases.

Our experiments with laurdan have indicated that TCS does affect fluidity of the lecithin membrane (Fig. 3A and B), although changes were opposite to those reported earlier for synthetic lipid systems with the temperature of the main phase transition above 0°C. In those systems TCS decreased phase transition temperature, disordering the bilayer and making it more fluid [19,20]. In our case, liposomes were composed of lecithin: a mixture of natural phosphatidylcholines with a high content of unsaturated fatty acids. The point of main phase transition of such a mixture lies below 0°C and, according to laurdan GP data, TCS decreased fluidity of lecithin membranes. We also found that for TCS-containing lecithin liposomes, GP-vs-λ_{ex} line was an ascending function, which is generally interpreted in the literature as an indication of phase heterogeneity. These results are in accordance with the data reported earlier that under certain conditions, incorporation of TCS into 1,2-dielaidoyl-sn-glycero-3-phosphoethanolamine liposomes induced formation of non-bilayer H(II) hexagonal phase, as well as appearance of new immiscible phases [20]. The formation of non-bilayer lipid/TCS aggregates could also explain the observations made in our DLS experiments: the emergence of a population of light-scattering particles whose size was substantially smaller than the size of liposomes (table 1).

Thus, permeabilization of SRB-loaded liposomes that we observed in our experiments might be a result of structural rearrangements following the formation of TCS-enriched domains in the membrane and non-bilayer phase transitions. Such rearrangements could result in the formation of transient pores in the lipid bilayer, and it should be noted that appearance of pores in the membrane after the addition of TCS (17 mol%) was predicted earlier by a computer simulation [45]. TCS molecules were found to be incorporated into the membrane perpendicular to phospholipids, which would result in the perturbation and disordering of the bilayer [21]. The disordered regions might undergo a polymorphic phase transition into non-bilayer structures, which would be accompanied by a temporal loss of membrane integrity.

Our experiments also show that permeabilization of liposomes by TCS is a pH-dependent process. As shown in Fig. 2, the effect of TCS is more pronounced at acidic pH values. The pKa of TCS is 8.14 [1], meaning that below pH 8.0, TCS is predominantly in the protonated, neutral form. Therefore, it is the neutral form of TCS that is the primary acting agent in the effects considered. The higher effectiveness of TCS at neutral pH may relate to partitioning of TCS molecules between the aqueous and lipid phases or to the potency of charged and uncharged TCS forms to induce polymorphic phase transitions.

As mentioned above, TCS has a pronounced effect on eukaryotic organisms. To a large extent, this effect is attributed to the TCS action in mitochondria [46,47]. It has been shown that micromolar concentrations of TCS cause a mitochondrial dysfunction, which can lead to cell death [14]. As demonstrated in the present work, the toxic effect of TCS may be underlain by permeabilization of the inner mitochondrial membrane. TCS is capable to provoke a high-amplitude swelling of rat liver mitochondria (Fig. 4), and we have confirmed that this is, indeed, a swelling, not an aggregation of the organelles (Fig. 6). The TCS-induced mitochondrial swelling is insensitive to the well-known specific inhibitor of the MPT pore opening, CsA (Fig. 7A), indicating a different mechanism of mitochondrial membrane permeabilization.

The results of our experiments also show that the rate and amplitude of swelling of rat liver mitochondria are higher in the presence of respiratory substrates (Fig. 7B). Perhaps, being an uncoupler, TCS accumulates in the energized organelles on the matrix side of the inner mitochondrial membrane. It is possible that this would facilitate permeabilization of mitochondria. The effect of TCS is especially pronounced when succinate is used as a respiratory substrate. TCS is an inhibitor of complex II of the respiratory chain [48], which can stop the uncoupling TCS cycle and lead to the local accumulation of TCS on the matrix side of the inner membrane. This issue, however, needs further clarification.

As shown in the present work, the TCS-induced swelling of mitochondria is severe enough to cause the release of cytochrome *c* from the organelles (Fig. 5). It can, therefore, be assumed that the release of this proapoptotic protein and the subsequent initiation of programmed cell death is one of the key mechanisms of the toxic effect of TCS on eukaryotic cells. It is important to note that, according to the literature data and our preliminary experiments on rat erythrocytes (data not shown), TCS is able to permeabilize the plasmatic membrane of eukaryotic cells in the concentration range of 10^{-5}–10^{-4} M. Recent studies have also demonstrated that at moderate concentrations, TCS induces influx of Ca^{2+} into cells [42] and increases production of reactive oxygen species [42, 48]. Together, these processes can result in depolarization of the plasmatic membrane, which – in combination with an increased level of Ca^{2+} in the cytoplasm – would be another factor facilitating permeability transition in

mitochondria. Thus, both the direct effect of TCS on mitochondria and its indirect influence on the organelles, via permeabilization of the plasmatic membrane, may contribute to the same mechanism of TCS toxicity: triggering cell death through the mitochondria-related pathway.

Thus, the results obtained show that TCS is a powerful agent capable to increase nonspecific permeability of both artificial (lecithin) and natural (mitochondrial) membranes. It can be supposed that the mechanism of the TCS effect on membrane permeability relates to its ability to disorder lipid bilayer and cause polymorphic phase transitions in the membrane. This mechanism may underlie not only permeabilization of liposomes but also the TCS-induced permeability transition in the mitochondrial membrane. Thus, in addition to other harmful effects in mitochondria – uncoupling of respiration and oxidative phosphorylation and inhibition of complex II of the respiratory chain – TCS can also induce the release of proapoptotic proteins from the organelles and trigger programmed cell death, which may be an essential component of the toxic effect of TCS on eukaryotic cells.

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References

[1] G.S. Dhillon, S. Kaur, R. Pulicharla, S.K. Brar, M. Cledón, M. Verma, R.Y. Surampalli, Triclosan: Current Status, Occurrence, Environmental Risks and Bioaccumulation Potential, Int. J. Environ. Res. Public Health. 12 (2015) 5657-5684. DOI: 10.3390/ijerph120505657

[2] C.W. Levy, A. Roujeinikova, S. Sedelnikova, P.J. Baker, A.R. Stuitje, A.R.Slabas, D.W. Rice, J.B. Rafferty, Molecular basis of triclosan activity, Nature. 398 (1999) 383–384. DOI: 10.1038/18803

[3] R.J. Heath, J.R. Rubin, D.R. Holland, E. Zhang, M.E. Snow, C.O. Rock, Mechanism of triclosan inhibition of bacterial fatty acid synthesis, J. Biol. Chem. 274 (1999) 11110–11114. DOI: 10.1074/jbc.274.16.11110.

[4] H.R. Lee, K.A. Hwang, K.H. Nam, H.C. Kim, K.C. Choi, Progression of breast cancer cells was enhanced by endocrine-disrupting chemicals, triclosan and octylphenol, via an estrogen receptor-dependent signaling pathway in cellular and mouse xenograft models. Chem Res Toxicol. 27(5) (2014) 834-842. DOI: 10.1021/tx5000156.

[5] K. Kolsek, M.L. Gobec, I. Mlinaric Rascan, M. Sollner Dolenc, Screening of bisphenol A, triclosan and paraben analogues as modulators of the glucocorticoid and androgen receptor activities, Toxicol. In Vitro 29 (2015) 8–15. DOI: 10.1016/j.tiv.2014.08.009.

[6] H. Huang, G. Du, W. Zhang, J. Hu, Di. Wu, L. Song, Y. Xia, X. Wang, The in vitro estrogenic activities of triclosan and triclocarban, J. Appl. Toxicol. 34(9) (2014) 1060-1067. DOI: 10.1002/jat.30012.

[7] R. Arancibia, M. Caceres, J. Martinez, P.C. Smith, Triclosan inhibits tumor necrosis factor-α-stimulated urikinase production in humangingival fibroblasts, J. Periodontol. 44 (2009) 726–735. DOI: 10.1111/j.1600-0765.2008.01184.x

[8] G. Cherednichenko, R. Zhang, R.A. Bannister, V. Timofeyev, N. Li, E.B. Fritsch, W. Feng, G.C. Barrientos, N.H. Schebb, B.D. Hammock, K.G. Beam, N. Chiamvimonvat, I.N. Pessah, Triclosan impairs excitation-contraction coupling and Ca2+ dynamics in striated muscle. Proc Natl Acad Sci USA. 109(35) (2012) 14158-14163. DOI: 10.1073/pnas.1211314109

[9] Z.A. Hassan, M.R. Abd El-Haleem, G.N. Mansour, Effect of Triclosan on the Renal Cortex of Adult Male Albino Rats and the Possible Protective Role of Ellagic Acid: Histological and Biochemical Study J Cytol Histol. 5 (2014), 285. DOI:10.4172/2157-7099.1000285

[10] K.C. Ahn, B. Zhao, J. Chen, G. Cherednichenko, E. Sanmarti, M.S. Denison, B. Lasley, I.N. Pessah, D. Kultz, D.P. Chang, S.J. Gee, B.D. Hammock,. In vitro biologic activities

of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: receptor-based bioassay screens. Environ. Health Perspect. 116(9) (2008) 1203–1210. DOI: 10.1289/ehp.11200.

[13] E.B. Fritsch, R.E. Connon, I. Werner, R.E. Davies, S. Beggel, W. Feng, I.N. Pessah, Triclosan impairs swimming behavior and alters expression of excitation-contraction coupling proteins in fathead minnow (Pimephales promelas), Environ. Sci. Technol. 47(4) (2013) 2008-2017. DOI: 10.1021/es303790b.

[14] C. Ajao, M.A. Andersson, V.V. Teplova, S. Nagy, C.G. Gahmberg, L.C. Andersson, M. Hautaniemi, B. Kakasi, M. Roivainen, M. Salkinoja-Salonen, Mitochondrial toxicity of triclosan on mammalian cells, Toxicology Report. 2 (2015) 624–637. DOI: 10.1016/j.toxrep.2015.03.012.

[15] A.P. Newton, S.M. Cadena, M.E. Rocha, E.G. Carnieri, M.B. Martinelli de Oliveira, Effect of triclosan (TRN) on energy-linked functions of rat liver mitochondria. Toxicol Lett. 160 (2005) 49-59. DOI: 10.1016/j.toxlet.2005.06.004.

[16] L.M. Weatherly, J. Shim, H.N. Hashmi, R.H. Kennedy, S.T. Hess, J.A. Gosse, Antimicrobial agent triclosan is a proton ionophore uncoupler of mitochondria in living rat and human mast cells and in primary human keratinocytes, J. Appl. Toxicol. 36(6) (2015) 777-789. DOI:10.1002/jat.3209.

[17] M.F. Yueh, K. Taniguchi, S. Chen, R.M. Evans, B.D. Hammock, M. Karin, R.H. Tukey, The commonly used antimicrobial additive triclosan is a liver tumor promoter, Proc. Natl. Acad. Sci. USA. 111 (2014) 17200-17205. DOI: 10.1073/pnas.1419119111.

[18] H.L. Zuckerbraun, H. Babich, R. May, M.C. Sinensky, Triclosan: cytotoxicity, mode of action, and induction of apoptosis in human gingival cells in vitro, Eur. J. Oral Sci. 106 (1998) 628-636. DOI: 10.1046/j.0909-8836.1998.eos106204.x.

[19] H. Lygre, G. Moe, R. Skålevik, H. Holmsen, Interaction of triclosan with eukaryotic membrane lipids, Eur J Oral Sci. 111(3) (2003) 216-222. DOI: 10.1034/j.1600-0722.2003.00034.x.

[20] J. Villalaín, C.R>Mateo, F.J. Aranda, S. Shapiro, V. Micol, Membranotropic effects of the antibacterial agent triclosan, Arch Biochem Biophys. 390(1) (2001) 128-136. DOI: 10.1006/abbi.2001.2356.

[21] J. Guillén, A. Bernabeu, S. Shapiro, J. Villalaín, Location and orientation of Triclosan in phospholipid model membranes, Eur Biophys J. 33(5) (2004) 448-53. DOI: 10.1007/s00249-003-0378-8.

[22] J. Regos, H.R. Hitz, Investigations on the mode of action of triclosan, a broad spectrum antibacterial agents, Zl Bakt Hyg. 226 (1974) 390–401. PMID: 4151803

[23] R.J. Heath, C.O. Rock, A triclosan-resistant bacterial enzyme, Nature. 406 (2000) 145–146. DOI: 10.1038/35018162.

[24] K.N. Belosludtsev, N.V. Belosludtseva, A.V. Agafonov, M.E. Astashev, A.S. Kazakov, N.-E.L. Saris, G.D. Mironova Ca^{2+}-dependent permeabilization of mitochondria and liposomes by palmitic and oleic acids: a comparative study, Biochim. Biophys. Acta. 1838(10) (2014) 2600-2606. DOI: 10.1016/j.bbamem.2014.06.017

[25] K. Belosludtsev, N.-E. Saris, L. Andersson, N. Belosludtseva, A. Agafonov, A. Sharma, D.A. Moshkov, G.D. Mironova, On the mechanism of palmitic acid-induced apoptosis: role of pore induced by palmitic acid and Ca^{2+} in mitochondria, J. Bioenerg. Biomembr. 38 (2006) 113-120. DOI: 10.1007/s10863-006-9010-9

[26] K.N. Belosludtsev, N.-E.L. Saris, N.V. Belosludtseva, A.S. Trudovishnikov, L.D. Lukyanova, G.D. Mironova, Physiological aspects of the mitochondrial cyclosporin A-insensitive palmitate/Ca^{2+}-induced pore: tissue specificity, age profile and dependence on the animal adaptation to hypoxia, J. Bioenerg. Biomembr. 41(4) (2009) 395-401. DOI: 10.1007/s10863-009-9230-x.

[27] K.N. Belosludtsev, N.V. Belosludtseva, G.D. Mironova, Possible mechanism for formation and regulation of the palmitate-induced cyclosporin A-insensitive mitochondrial pore, Biochemistry (Mosc.). 70 (2005) 815–821. DOI: 10.1007/s10541-005-0189-x.

[28] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193(1) (1951) 265-275. http://www.jbc.org/content/193/1/265.citation

[29] F. Appaix, M. Minatchy, C. Riva-Lavieille, J. Olivares, B. Antonsson, V.A. Saks, Rapid spectrophotometric method for quantitation of cytochrome c release from isolated mitochondria or permeabilized cells revisited. Biochim. Biophys. Acta. 1457(3) (2000) 175-181. DOI: 10.1016/S0005-2728(00)00098-0

[30] A. Agafonov, E. Gritsenko, K. Belosludtsev, A. Kovalev, O. Gateau-Roesch, N.-E.L. Saris, G.D. Mironova, A permeability transition in liposomes induced by the formation of Ca^{2+}/palmitic acid complexes, Biochim. Biophys. Acta. 1609(2) (2003) 153-160. DOI: 10.1016/S0005-2736(02)00666-1.

[31] T. Parasassi, E.K. Krasnowska, L. Bagatolli, E. Gratton, Laurdan and Prodan as Polarity-Sensitive Fluorescent Membrane Probes, J. Fluoresc. 8(4) (1998) 365−373. DOI: 10.1023/A:1020528716621.

[32] T. Parasassi, G. Stasio, A. d'Ubaldo, E. Gratton, Phase Fluctuation in Phospholipid Membranes Revealed by Laurdan Fluorescence, Biophys. J. 57 (1990) 1179−1186. DOI: 10.1016/S0006-3495(90)82637-0.

[33] M.V. Dubinin, M.E. Astashev, N.V. Penkov, S.V. Gudkov, I.A. Dyachenko, V.N. Samartsev, K.N. Belosludtsev, Effects of Phospholipase A_2 Inhibitors on Bilayer Lipid Membranes, J. Membr. Biol. 249(3) (2016) 339-347. DOI: 10.1007/s00232-016-9872-7.

[34] L.A. Bagatolli, T. Parasassi, G.D. Fidelio, E. Gratton, A model for the interaction of 6-lauroyl-2-(N,N-dimethylamino)naphthalene with lipid environments: Implications for spectral properties, Photochem Photobiol 70 (1999) 557–564. DOI: 10.1111/j.1751-1097.1999.tb08251.x.

[35] T. Parasassi, E. Gratton, Membrane lipid domains and dynamics as detected by Laurdan fluorescence, J Fluorescence. 5 (1995) 59–69. DOI: 10.1007/BF00718783.

[36] T. Baumgart, G. Hunt, E.R. Farkas, W.W. Webb, G.W. Feigenson, Fluorescence probe partitioning between Lo/Ld phases in lipid membranes. Biochim Biophys Acta. 1768 (2007) 2182–2194. DOI: 10.1016/j.bbamem.2007.05.012.

[37] S. Mukherjee, A. Chattopadhyay, Monitoring the organization and dynamics of bovine hippocampal membranes utilizing Laurdan generalized polarization, Biochim Biophys Acta. 1714 (2005) 43–55. DOI: 10.1016/j.bbamem.2005.06.010.

[38] O. Wesołowska, K. Michalak, A.B. Hendrich, Direct visualization of phase separation induced by phenothiazine-type antipsychotic drugs in model lipid membranes, Mol Membr. Biol. 28(2) (2011) 103-114. DOI: 10.3109/09687688.2010.533706.

[39] V.V. Lemeshko, L.F. Lopez, S. Solano, R. Torres, The natural antioxidant otobaphenol delays the permeability transition of mitochondria and induces their aggregation, Antioxid. Redox. Signal. 5(3) (2003) 281-290. DOI: 10.1089/152308603322110869.

[40] V.V. Lemeshko, Redox state-dependent aggregation of mitochondria induced by cytochrome c, Mol. Cell Biochem. 360(1-2) (2012) 111-119. DOI: 10.1007/s11010-011-1049-1.

[41] V.V. Lemeshko, S. Solano, L.F. López, D.A. Rendon, P. Ghafourifar, L.A. Gomez, Dextran causes aggregation of mitochondria and influences their oxidoreductase activities and light scattering, Arch. Biochem. Biophys. 412(2) (2003) 176-185. DOI: 10.1016/S0003-9861(03)00034-1.

[42] M. González-Pleiter, C. Rioboo, M. Reguera, I. Abreu, F. Leganes, A. Cid, F. Fernández-Piñas, Calcium mediates the cellular response of Chlamydomonas reinhardtii to the emerging aquatic pollutant Triclosan. Aquat. Toxicol. 186 (2017) 50-66. DOI: 10.1016/j.aquatox.2017.02.021.

[43] M. Adolfsson-Erici, M. Pettersson, J. Parkkonen, J. Sturve, Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden, Chemosphere. 46(9-10) (2002) 1485-14859. DOI: 10.1016/S0045-6535(01)00255-7.

[44] H. Singer, S. Muller, C. Tixier, L. Pillonel, Triclosan: Occurrence and Fate of a Widely Used Biocide in the Aquatic Environment: Field Measurements in Wastewater Treatment Plants, Surface Waters, and Lake Sediments, Environ. Sci. Technol. 36(23) (2002) 4998–5004. DOI: 10.1021/es025750i/

[45] M. Orsi, M.G. Noro, J.W. Essex, Dual-resolution molecular dynamics simulation of antimicrobials in biomembranes, J R Soc Interface. 8(59) (2011) 826-41. DOI: 10.1098/rsif.2010.0541.

[46] M. Porceddu, N. Buron, C. Roussel, G. Labbe, B. Fromenty, A. Borgne-Sanchez, Prediction of liver injury induced by chemicals in human with a multiparametric assay on isolated mouse liver mitochondria, Toxicol. Sciences. 129 (2012) 332–345. DOI: 10.1093/toxsci/kfs197.

[47] J.N. Meyer, M.C. Leung, J.P. Rooney, A. Sendoel, M.O. Hengartner, G.E. Kisby, A.S. Bess, Mitochondria as a target of environmental toxicants, Toxicol Sci. 134 (2013) 1-17. DOI: 10.1093/toxsci/kft102.

[48] V.V. Teplova, K.N. Belosludtsev, A.G. Kruglov, Mechanism of triclosan toxicity: Mitochondrial dysfunction including complex II inhibition, superoxide release and uncoupling of oxidative phosphorylation, Toxicol. Lett. 275 (2017) 108-117. DOI: 10.1016/j.toxlet.2017.05.004.

FIGURE LEGENDS

Fig. 1. Effect of TCS on the release of the fluorescent dye SRB from lecithin unilammelar liposomes. (A) Fluorescence changes in the suspension of SRB-loaded LUV after the addition of TCS. The incubation medium contained 10 mM Tris-HCl (pH 7.5), 50 μM EGTA, and 40 mM KCl. Additives: LUV (40–45 μM), 26 μM TCS, 0.1% TX-100. (B) Dependence of the release of SRB from lecithin liposomes on the concentration of TCS. Mean values ± SD are represented (n = 4).

Fig. 2. Effect of pH on TCS-induced SRB release from LUV. The release of SRB from liposomes was induced by 34 μM TCS. Experimental conditions were as in Fig. 1. Mean values ± SD are represented (n = 5). * $p < 0.05$ compared to pH 7.5.

Fig. 3. Effect of TCS on laurdan GP in egg-PC liposomes. (A) Laurdan emission spectra (λ_{ex} = 360 nm) at different concentrations of TCS (0, 17, 34, 51 and 68 μM). The insert shows the dependence of calculated GP values on the concentration of TCS. (B) Effect of TCS on laurdan GP in lecithin liposomes depending on λ_{ex}: 1, LUV; 2, LUV + 34 μM TCS. Mean values ± SD are represented (n = 3). The incubation medium contained 10 mM Tris-HCl (pH 7.5), 50 μM EGTA, and 40 mM KCl. Measurements were performed at 37°C**.**

Fig. 4. TCS-induced swelling of rat liver mitochondria. (A) Swelling of rat mitochondria (0.4 mg/mL), measured by absorbance at 540 nm (A_{540}), was induced by 34 μM TCS in 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 5 μM EGTA, 1 μM rotenone, and 10 mM Hepes / KOH buffer, pH 7.4. (B) Dependence of the rate of swelling of rat liver mitochondria on the concentration of TCS. Mean values ± SD are represented (n = 7). (C) Electron micrographs of rat liver mitochondria before (control) and after (TCS) treatment with 51 μM TCS.

Fig. 5. TCS induces the release of cytochrome c from mitochondria. Mitochondria were incubated under the same conditions used in the swelling experiments (Fig. 4). Mean values ± SD are represented (n = 3). * $p < 0.05$ compared to control (without TCS).

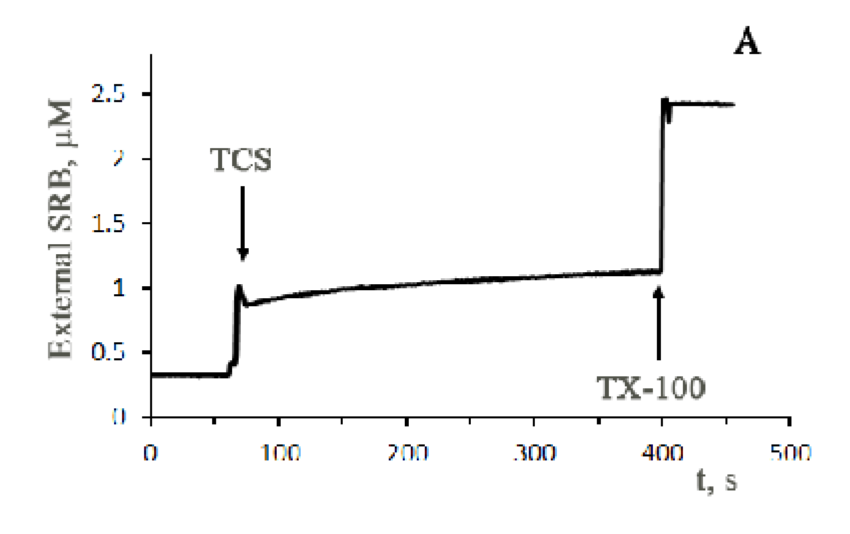
Fig. 6. A confocal microscopy examination of the mitochondrial suspension in the absence (A) and presence (B) of 51 μM TCS using MitoTracker™ Red. The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 5 μM EGTA, 1 μM rotenone, 0.5 μM MitoTracker™ Red and 10 mM Hepes / KOH buffer, pH 7.4.

Fig. 7. Induction of mitochondrial swelling by TCS under different conditions. Swelling of rat liver mitochondria was induced with 34 μM TCS. The swelling rate was expressed as a percentage of the mean swelling rate recorded in a series of experiments with 34 μM (10 μg/mL) TCS. (A) Effect of 1 μM CsA on the TCS-induced swelling of rat liver mitochondria. The medium contained 210 mM mannitol, 70 mM sucrose, 5 μM EGTA, 1 μM rotenone, and 10 mM Hepes / KOH buffer, pH 7.4. Mean values ± SD are represented (n = 5). (B) TCS-induced swelling of rat liver mitochondria in the absence and presence of respiratory substrates: 5 mM succinate or 2.5 mM glutamate plus 2.5 mM malate. The medium contained 210 mM mannitol, 70 mM sucrose, 5 μM EGTA, 1 μM rotenone (only in the absence of respiratory substrates or in the presence of succinate), and 10 mM Hepes / KOH buffer, pH 7.4. Mean values ± SD are represented (n = 4). * $p < 0.05$ compared to succinate.

Table 1. Effect of TCS on the hydrodynamic diameter of lecithin liposomes

TCS, μM	Peak 1, D, nm	Peak 2, D, nm
0	133.9±8.6	
17	131.6±8.7	
34	151.6±5.4*	
51	156.8±4.3* (8.9±1.4%)	59.8±8.3* (91.1±1.4%)

The incubation medium contained 10 mM Tris-HCl (pH 7.5), 50 μM EGTA, and 40 mM KCl. Values in parentheses indicate the percentage of particles in the system. Mean values ± SD are represented (n = 3). *p < 0.05 compared to control.


A
External SRB, µM
2.5
2
1.5
1
0.5
0
TCS
TX-100
0
100
200
300
400
500
t, s

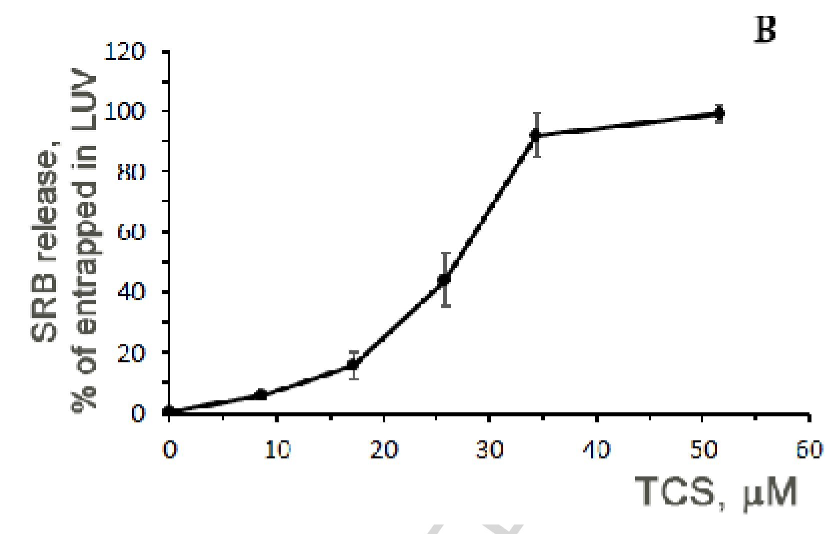
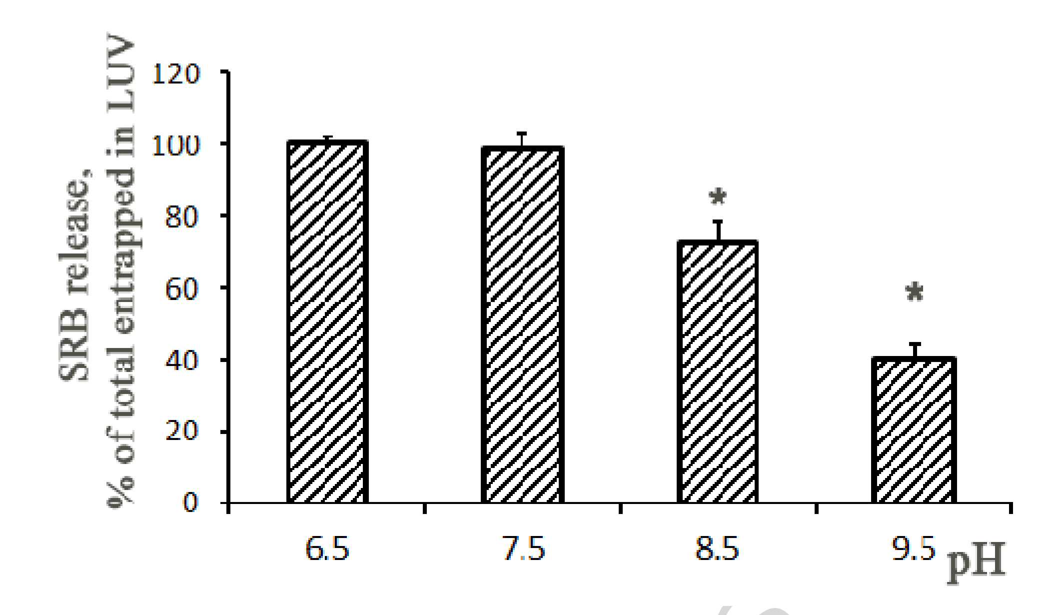

B
SRB release,
% of entrapped in LUV
120
100
80
60
40
20
0
10
20
30
40
50
60
TCS, µM

Figure 1



SRB release,
% of total entrapped in LUV
120
100
80
60
40
20
0
*
*
6.5
7.5
8.5
9.5
pH

Figure 2

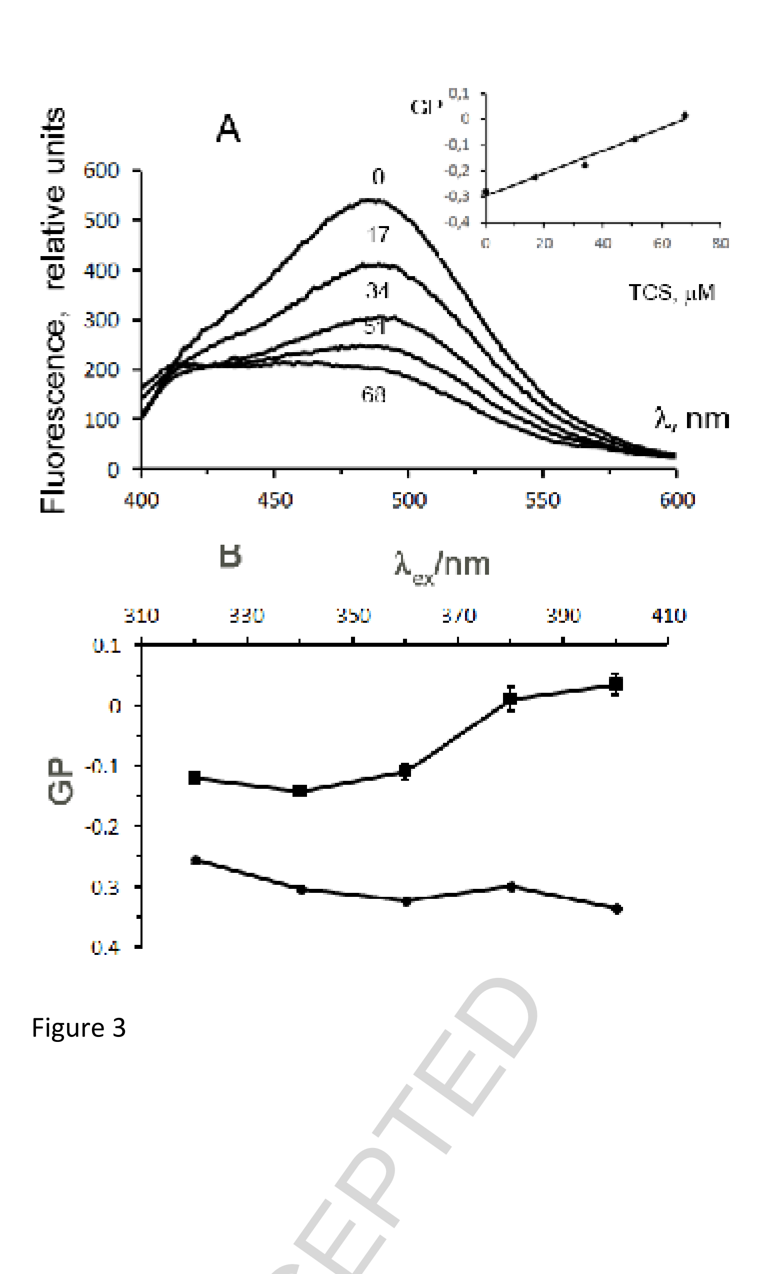
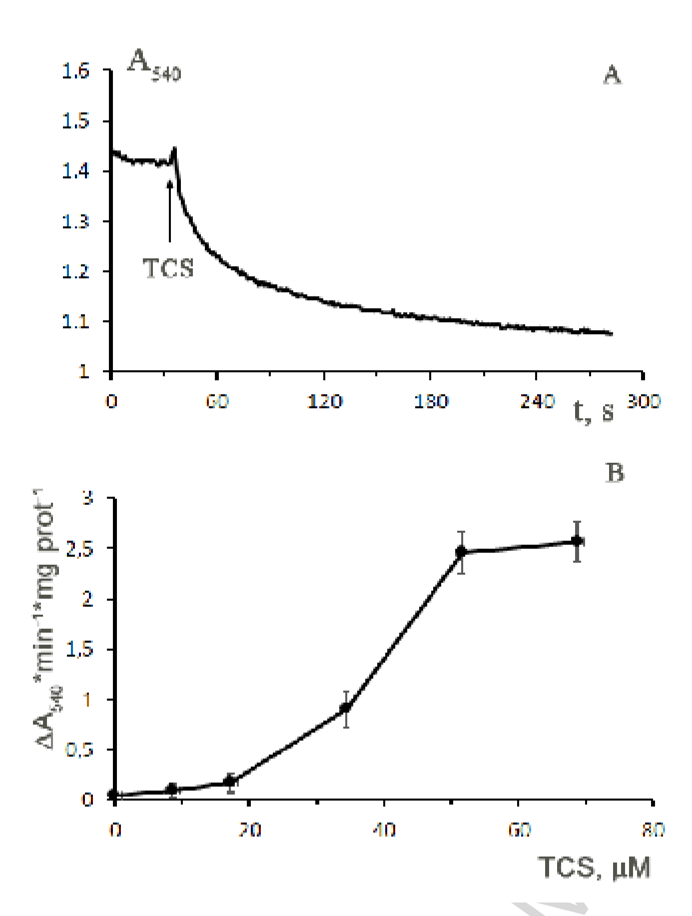

A
Fluorescence, relative units
600
500
400
300
200
100
0
400
450
500
550
600
λ, nm
0
17
34
51
68
GP
TCS, μM
B
λ_{ex}/nm
310
330
350
370
390
410
0.1
0
-0.1
-0.2
0.3
0.4
GP

Figure 3



A540
A
1.6
1.5
1.4
1.3
1.2
1.1
1
TCS
0
60
120
180
240
t, s
300
B
ΔA540*min-1*mg prot-1
3
2,5
2
1,5
1
0,5
0
0
20
40
60
80
TCS, µM

Figure 4ab

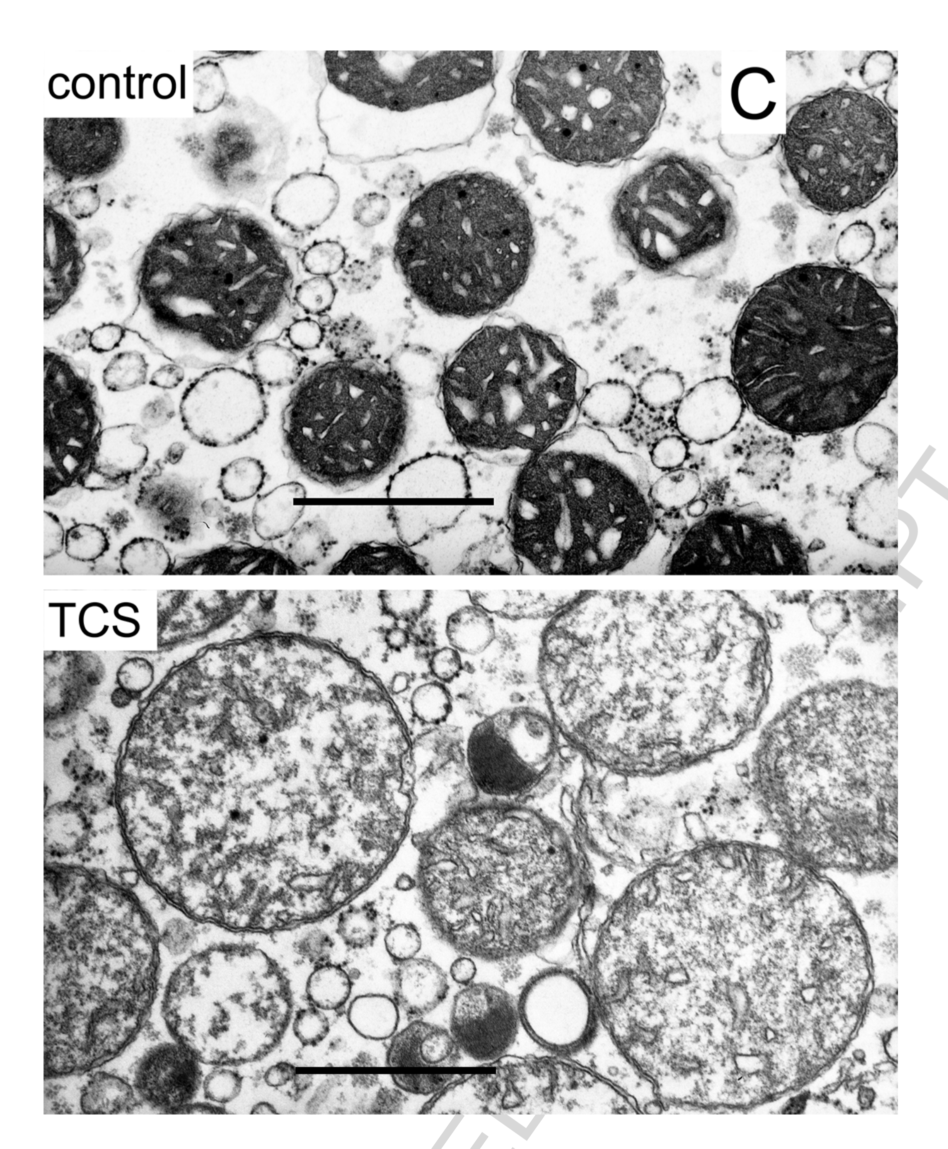


Figure 4c

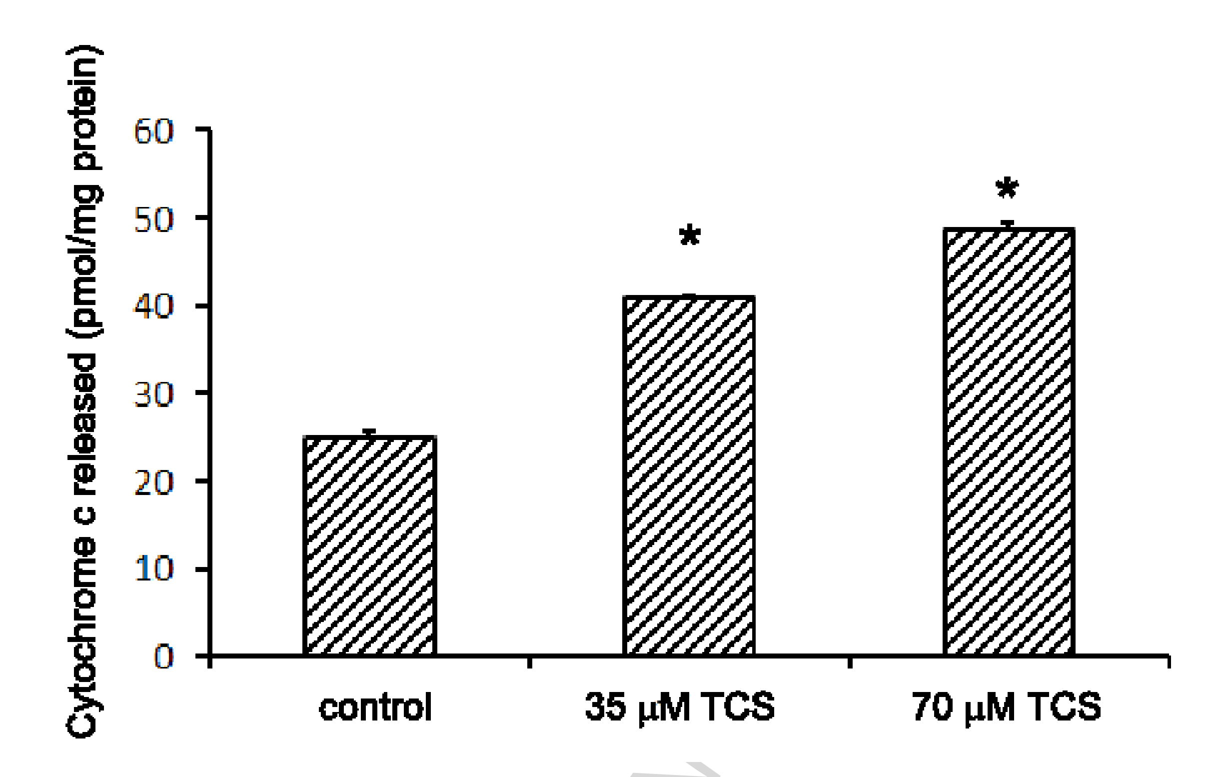

Cytochrome c released (pmol/mg protein)
60
50
40
30
20
10
0
*
*
control
35 µM TCS
70 µM TCS

Figure 5

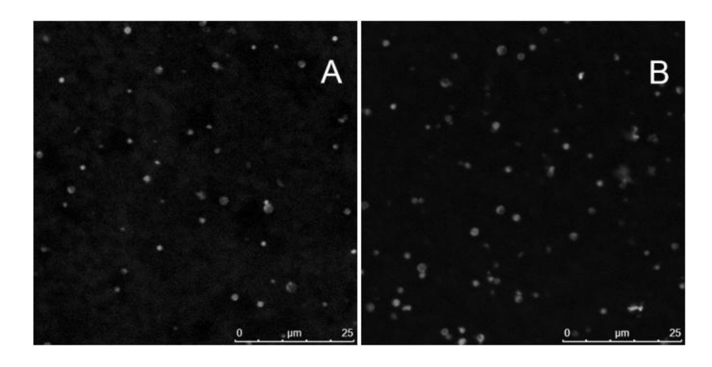
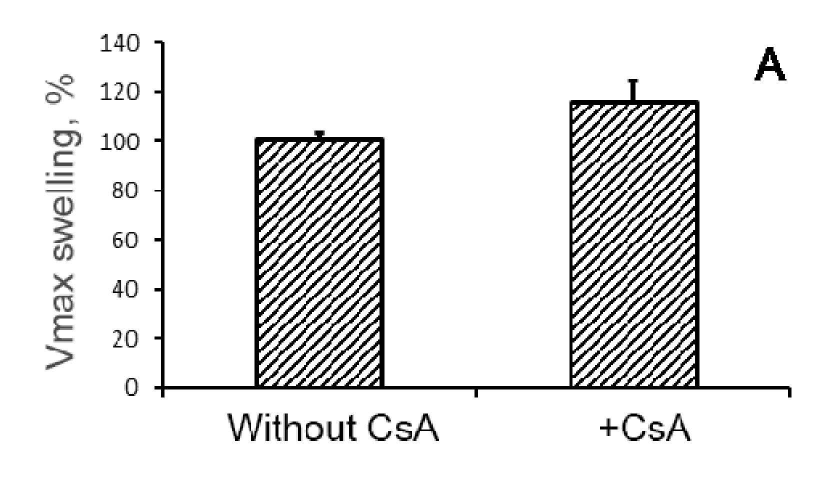


Figure 6




A
Vmax swelling, %
140
120
100
80
60
40
20
0
Without CsA
+CsA

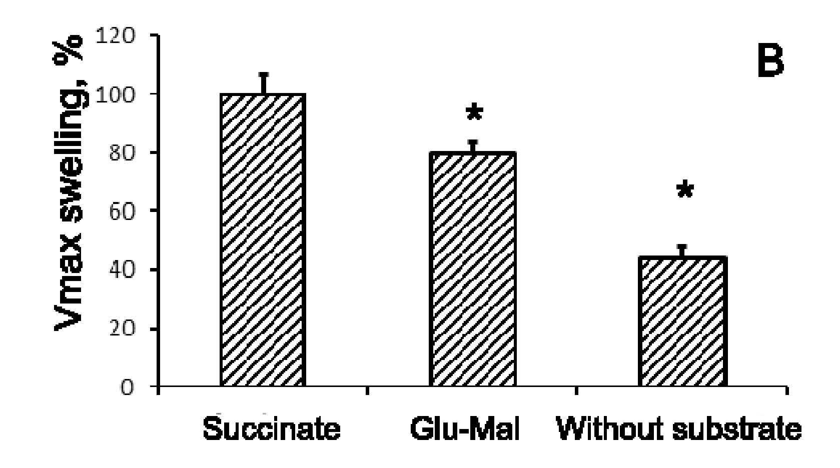
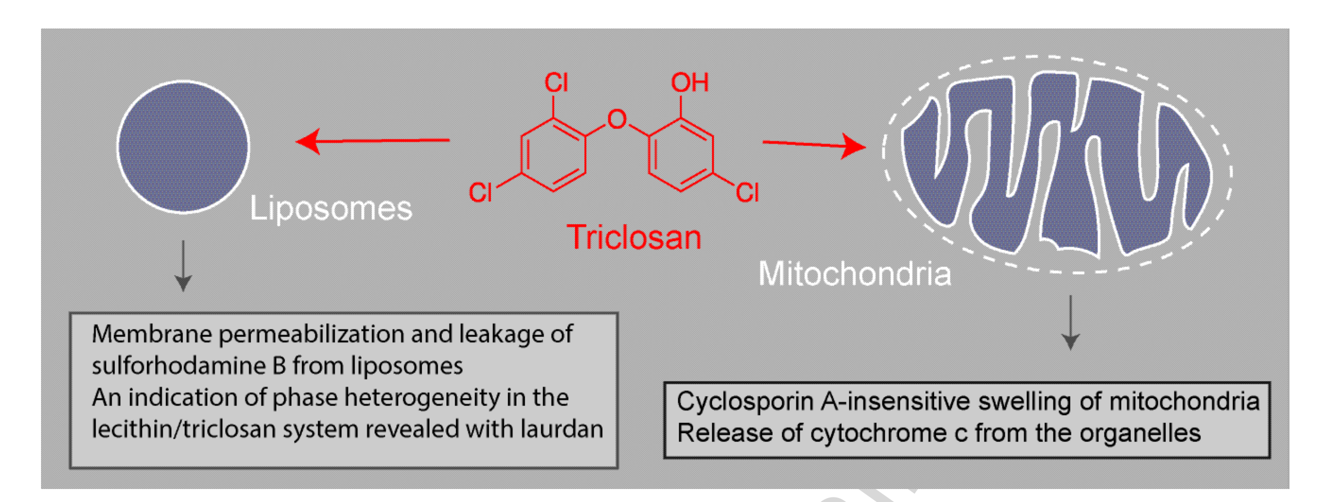

B
Vmax swelling, %
120
100
80
60
40
20
0
*
*
Succinate
Glu-Mal
Without substrate

Figure 7

Graphical abstract



Highlights

Triclosan is able to permeabilize the membrane of lecithin liposomes.

Raising pH of the medium inhibits the triclosan-induced liposome permeabilization

Laurdan fluorescence data indicate a phase heterogeneity in triclosan/lecithin system

Triclosan promotes a cyclosporin A-insensitive swelling of rat liver mitochondria.

Triclosan induces cytochrome c release from rat liver mitochondria

